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14. ABSTRACT A large body of data suggests that selenium supplementation may be used as a chemopreventive strategy to reduce the risk of prostate cancer. In spite of this, little is known regarding the use of selenium as a cancer therapy. High doses of selenite can deplete cells of the primary intracellular antioxidant, glutathione, and generate superoxide. The net effect of the metabolism of selenite is a profound alteration in the cellular redox status and generation of potentially lethal reactive oxygen species. We have characterized the tumor-selective killing properties of selenite in patient-matched pairs of normal and malignant prostate cells and demonstrated the ability of selenite to sensitize prostate cancer cells to γ -irradiation. Currently, we are examining the effects of selenite on androgen receptor signaling <i>in vitro</i> and <i>in vivo</i> . The primary goal of this proposal was to generate pre-clinical data supporting the concept that selenite might be a novel chemotherapeutic agent for prostate cancer.					
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Introduction

The anti-tumor activities of selenium compounds are dependent upon the dose and chemical form. The inorganic form of selenium, selenite (SeO_3^{2-}), undergoes thiol-dependent reduction to hydrogen selenide (H_2Se) (1). Hydrogen selenide can supply selenium for the synthesis of selenoproteins or undergo sequential enzymatic methylation to yield mono-, di-, and tri-methylated metabolites. The further oxidative metabolism of hydrogen selenide can also produce superoxide anions. At lower concentrations, the major effects of selenite are related to its role as a micronutrient. However at higher concentrations, selenite metabolism depletes cells of the primary intracellular antioxidant, glutathione (GSH), and generates reactive oxygen species (Fig. 1). Alterations in the intracellular redox state can affect the activity of redox-sensitive proteins via the oxidation of critical cysteine residues, which may in turn have downstream effects on signal transduction and gene transcription. Furthermore, excessive production of reactive oxygen species can overwhelm the buffering capacity of a cell and induce apoptosis (2).

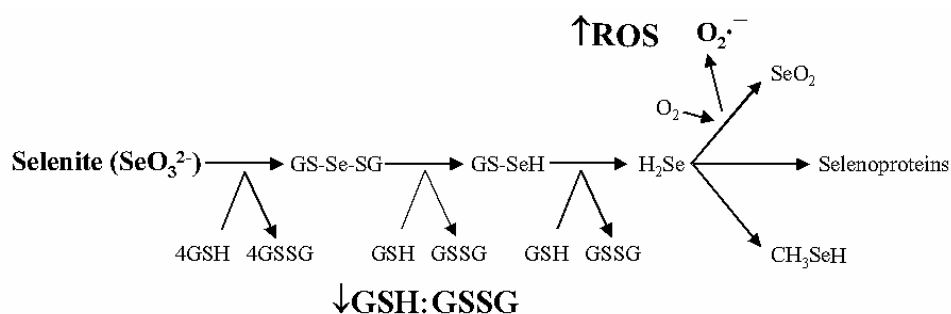


Figure 1 Schematic illustration showing the metabolic reduction of selenite with GSH.

We have characterized the response of prostate cancer cell lines and patient-matched pairs of normal and malignant prostate cells to selenite. Selenite showed tumor-selective killing which correlated with changes in Bcl-2 family member expression, altered intracellular GSH status, and MnSOD expression. We also showed the ability of selenite to sensitize prostate cancer cells to γ -irradiation. Our current work has focused on the ability of selenite to inhibit prostate cancer growth *in vitro* and *in vivo* through the inhibition of androgen receptor signaling. We are also working on combining selenite with radiation therapy *in vivo* using the LAPC-4 xenograft model. Our goal is to generate preclinical data supporting the concept that selenite might be useful as a novel chemotherapeutic agent alone or in combination with radiation therapy to treat prostate cancer.

Body

Task 1. Study the effects of selenite on apoptosis and cell survival in LAPC-4 cells and primary prostate cancer cell strains *in vitro* and *in vivo*.

Background

The androgen receptor (AR) not only plays an important role in the development of androgen-dependent prostate cancer, but is also present and active in hormone-refractory disease (3). Androgen binding stimulates AR translocation to the nucleus where it interacts with specific androgen-responsive elements (ARE) on the promoters of target genes involved in the proliferation and differentiation of prostate cells. Androgen deprivation continues to be the standard therapy for advanced and metastatic prostate cancer. Although prostate cancer initially responds to androgen withdrawal, a majority of these cancers eventually progress to a hormone-refractory state with a potentially fatal outcome. Several AR related mechanisms influence the development of hormone-refractory prostate cancer (4). Increased AR expression, mutations in the AR ligand binding domain, and ligand-independent activation of the AR may allow prostate cancer to progress in an androgen-deprived environment. Therefore, novel therapies that target the AR and its regulatory pathways have significant implications for prostate cancer prevention and the treatment of neoplastic disease.

Recently methylseleninic acid (MSeA, $\text{CH}_3\text{SeO}_2\text{H}$) has been reported to down-regulate prostate-specific antigen (PSA) expression via disruption of AR signaling in a number of prostate cancer cell lines (5,6). The metabolism of MSeA is different from selenite in that it bypasses the hydrogen selenide metabolite pool. Upon entering the cell, MSeA reacts directly with reduced GSH to produce methylselenol (CH_3SeH), the putative active selenium metabolite for cancer prevention (7). Our goal was to characterize the effects of selenite on AR signaling in prostate cancer cells and to determine whether selenite and MSeA shared similar molecular mechanisms of action.

Selenite inhibits LAPC-4 growth and AR and PSA expression

LAPC-4 human prostate cancer cells express a wild-type AR and respond to androgen with increased proliferation and increased expression and secretion of PSA. The effect of selenite on the proliferation of LAPC-4 cells was measured using the MTS assay. Fig. 2A shows the dose response of LAPC-4 cells to increasing concentrations of selenite for 24 hours. LAPC-4 cells treated with 2.5 μM selenite showed no change relative to control, however, cell proliferation was 72.9% and 55.4% of control after treatment with 5 μM or 10 μM selenite, respectively. We next tested whether the inhibition of cell growth by selenite was associated with decreased AR expression. First, we assessed the effects of selenite on the transcriptional activity of the AR promoter. LAPC-4 cells were transfected with an AR promoter-luciferase construct and then treated with selenite for 24 hours. Fig. 2B shows the dose-dependent inhibition of AR promoter activity by selenite. Importantly, decreased AR promoter activity was observed after treatment with 2.5 μM selenite, suggesting that the inhibition of AR transcription occurs before any decrease in cell number. The decrease in AR promoter driven luciferase activity after exposure to selenite for 24 hours was coupled to decreased AR protein levels as determined by Western blot analysis (Fig. 2C).

The AR is the most important regulatory factor for PSA gene transcription. To test whether the modulation of AR expression by selenite was associated with decreased PSA expression at the transcriptional level we measured AR and PSA mRNA quantitatively by real-time RT-PCR. LAPC-4 cells were treated with 10 μM selenite for various lengths of time and the results are shown in Fig. 2D. The expression of AR and PSA mRNA followed a similar time response pattern after dosing with selenite. Transcript levels decreased as early as 6 hours after exposure to selenite and the inhibition was approaching 100% after 24 hours.

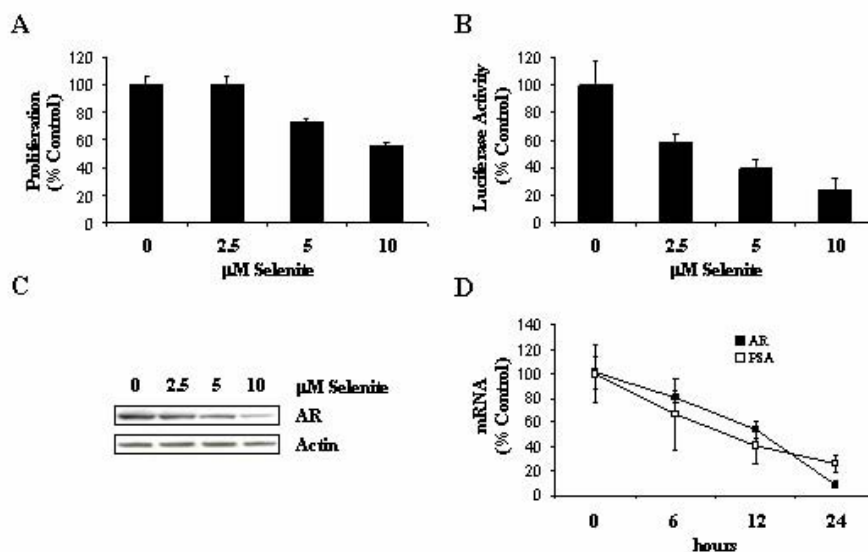


Figure 2 Effect of selenite on LAPC-4 cell proliferation and AR and PSA expression. **A**, LAPC-4 cells were treated with selenite at the indicated concentrations for 24 hours and cell proliferation was measured by MTS assay. **B**, LAPC-4 cells were co-transfected with the AR promoter-luciferase construct, pAR-luc, and pSV40-ren and then treated with selenite for 24 hours. Luciferase activity was normalized to renilla and expressed as percent of control. **C**, AR protein expression in LAPC-4 cells after exposure to selenite for 24 hours as detected by Western blot analysis. Actin protein expression was used to normalize for loading. **D**, LAPC-4 cells were treated with 10 μM selenite for 6, 12, and 24 hours and AR and PSA mRNA was measured by real-time RT-PCR. The expression of TBP was used for normalization. Values represent the mean \pm SD for 3 experiments.

Selenite interferes with R1881-induced PSA expression in LAPC-4 cells

The experiments described in Figure 2 were performed in cells cultured in 10% fetal bovine serum. We also tested the effects of selenite on PSA protein expression in LAPC-4 cells cultured in charcoal-stripped FBS with increasing concentrations of R1881, a potent synthetic androgen. Treatment of LAPC-4 cells with R1881 for 24 hours led to a dose-dependent increase in cellular PSA protein levels (Fig. 3A). Simultaneous treatment with 10 μM selenite inhibited the induction of PSA by R1881. An ELISA was performed to measure the amount of secreted PSA into the conditioned media from the same cells. Fig. 3B shows that selenite was also able to completely suppress R1881-induced PSA secretion.

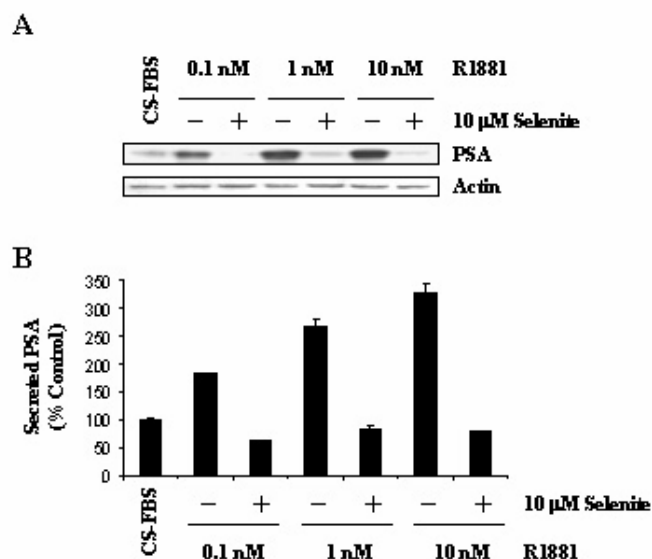


Figure 3 Selenite inhibits R1881-induced PSA expression. **A**, Increasing amounts of R1881 were added to LAPC-4 cells growing in hormone-depleted media and cellular PSA was detected by Western blot analysis 24 hours later. Actin protein expression was used to normalize for loading. **B**, ELISA detection of secreted PSA in the conditioned media from the same cells. PSA values were normalized to total protein per sample. Values represent the mean \pm SD for 3 experiments.

NAC attenuates selenite-induced down-regulation of AR and PSA

The pro-apoptotic activity of selenite is mainly dependent on its ability to deplete GSH and induce oxidative stress. We examined how modulation of intracellular GSH could influence selenite-induced down-regulation of AR expression and activity. LAPC-4 cells were pre-treated with 10 mM N-acetylcysteine (NAC) for 24 hours and exposed to selenite for an additional 24 hours. Pre-treatment with NAC increased intracellular GSH levels in LAPC-4 as much as 80% (data not shown). As shown in Fig. 4A, the addition of NAC blocked the decrease in AR protein levels following treatment with selenite. A radioligand binding assay was performed with [³H]-DHT to assess functional AR expression after treatment with selenite. Fig. 4B shows that pre-treatment with NAC restored functional AR levels to control after exposure to selenite. The effect of NAC on AR expression occurred at the transcriptional level. Using real-time RT-PCR we found that NAC was able to inhibit the down-regulation of AR mRNA by selenite (Fig. 4C). Consequently, NAC supplementation also maintained normal levels of PSA mRNA and secreted PSA in LAPC-4 cells treated with selenite (Fig. 4C and 4D).

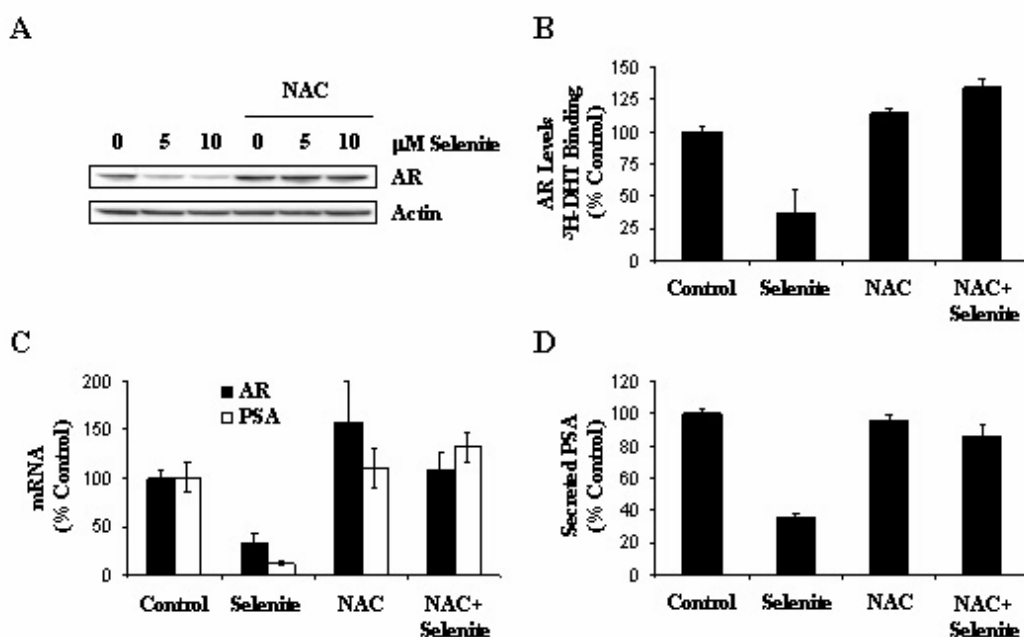


Figure 4 NAC inhibits selenite-induced down-regulation of the AR and PSA. LAPC-4 cells were pre-treated with 10 mM NAC for 24 hours and then treated with selenite for another 24 hours. **A**, AR protein expression determined by Western blot analysis after exposure to 5 or 10 μ M. Actin protein expression was used to normalize for loading. **B**, Functional AR levels measured by [³H]-DHT binding, **(C)** AR and PSA mRNA measured by real-time RT-PCR, and **(D)** ELISA detection of secreted PSA after exposure to 10 μ M selenite with or without NAC pre-treatment. Values represent the mean \pm SD for 3 experiments.

MSeA has previously been shown to inhibit AR expression and signaling in LAPC-4 and LNCaP prostate cancer cells. Since MSeA reacts with reduced GSH within the cell, we tested whether altering the intracellular GSH content with NAC could also modulate the effect of MSeA on the AR. LAPC-4 cells were treated with 10 mM NAC for 24 hours and then exposed to 10 μ M MSeA for another 24 hours. Fig. 5A shows that pre-treatment with NAC did not inhibit MSeA-induced down-regulation of AR protein levels. Similarly, NAC did not prevent the decrease in PSA secretion caused by MSeA in the same cells (Fig. 5B). We next tested the effect of NAC on selenite and MSeA-induced inhibition of AR and PSA expression in LNCaP prostate cancer cells, which express a mutant but functional AR, to determine the universality of this response. LNCaP cells were treated with 10 mM NAC for 24 hours and then dosed with 5 μ M selenite or MSeA for 24 hours. Selenite and MSeA decreased AR protein expression and PSA secretion in LNCaP cells, and NAC pre-treatment was again found to only block the inhibition caused by selenite, but not by MSeA (Fig. 5C, 5D, and 5E).

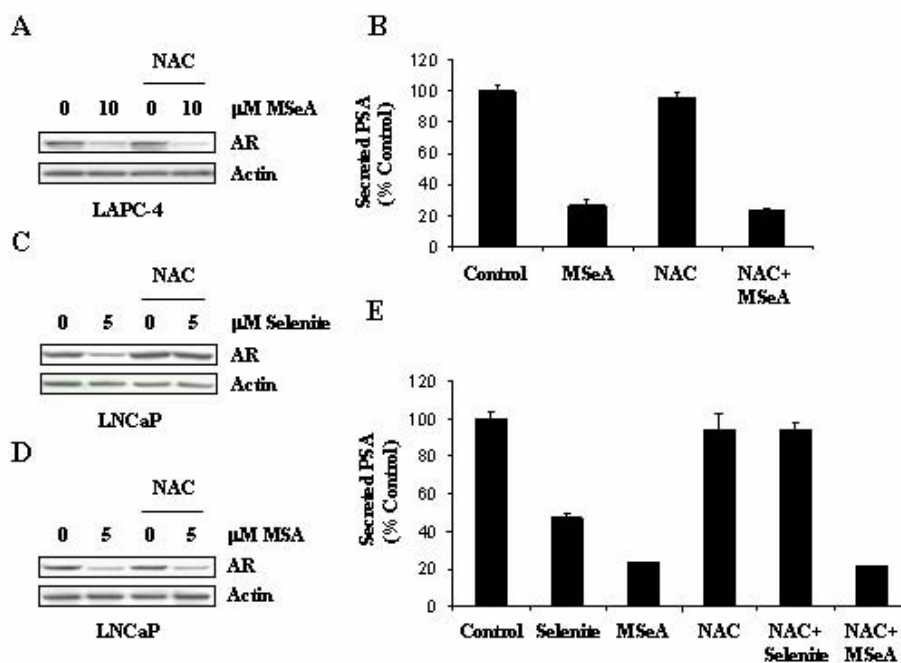


Figure 5 NAC does not inhibit MSeA-induced down-regulation of the AR and PSA. **A**, LAPC-4 cells were pre-treated with 10 mM NAC for 24 hours and then treated with 10 μ M MSeA for another 24 hours and AR protein expression was detected by Western blot analysis. **B**, ELISA detection of secreted PSA from the same cells. LNCaP cells were pre-treated with 10 mM NAC for 24 hours and then treated with 5 μ M selenite (**C**) or MSeA (**D**) for 24 hours and AR protein expression was detected by Western blot analysis. **E**, ELISA detection of secreted PSA from the same cells. Actin protein expression was used to normalize for loading. Values represent the mean \pm SD for 3 experiments.

Differential involvement of superoxide in selenite versus MSeA-mediated down-regulation of the AR

Superoxide produced as a result of selenite metabolism is an important mediator of selenite-induced apoptosis. We tested whether the inhibition of AR expression by selenite or MSeA in prostate cancer cells was mediated at least in part by superoxide. LAPC-4 and LNCaP cells were exposed to selenite or MSeA in the presence or absence of 5 μ M MnTMPyP for 24 hours and AR protein expression was measured by Western blot analysis. MnTMPyP is a stable manganese-porphyrin complex that has been shown to dismutate superoxide radicals to hydrogen peroxide. Fig. 6 shows that MnTMPyP was able to prevent the decrease in AR protein caused by selenite, but not by MSeA in both LAPC-4 and LNCaP cells. The data implicate a role for superoxide in the down-regulation of the AR by selenite, but not MSeA.

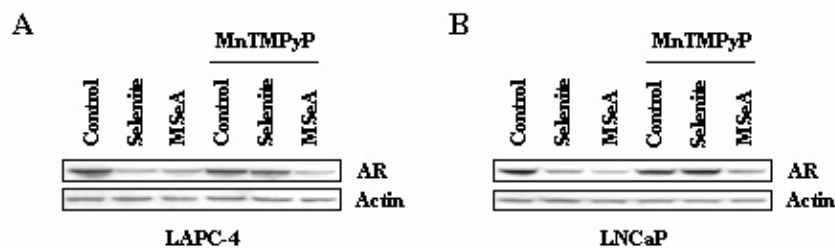


Figure 6 Role of superoxide in selenite and MSeA-induced inhibition of AR expression. LAPC-4 and LNCaP cells were treated with selenite or MSeA in the presence or absence of 5 μ M MnTMPyP. **A**, Western blot analysis of AR protein expression in LAPC-4 cells 24 hours after treatment with 10 μ M selenite or MSeA, and **(B)** LNCaP cells 24 hours after treatment with 5 μ M selenite or MSeA. Actin protein expression was used to normalize for loading.

Effects of selenite and MSeA on Sp1

The Sp1 transcription factor and its DNA binding motif play an important role in regulating the transcriptional activities of the AR promoter (8). Sp1 activity has been shown to be redox-sensitive. Therefore, we tested whether selenite had an effect on Sp1 activity. Using a Sp1-luciferase reporter vector we observed decreased Sp1 activity in LAPC-4 cells after 8 hours of exposure to selenite (Fig. 7A). Both LAPC-4 and LNCaP cells were then exposed to selenite or MSeA for 8 hours and Sp1 was measured in the nuclear extracts by Western blot analysis. As shown in Fig. 7B, selenite decreased Sp1 expression in both LAPC-4 and LNCaP cells, whereas MSeA did not. In addition, pre-treatment with NAC blocked the effect of selenite on nuclear Sp1 expression. The results indicate that reduced nuclear Sp1 expression leading to decreased Sp1 activity may be the mechanism by which selenite inhibits AR expression. A scheme summarizing the possible differential effects of selenite and MSeA on AR expression is shown in Fig. 8.

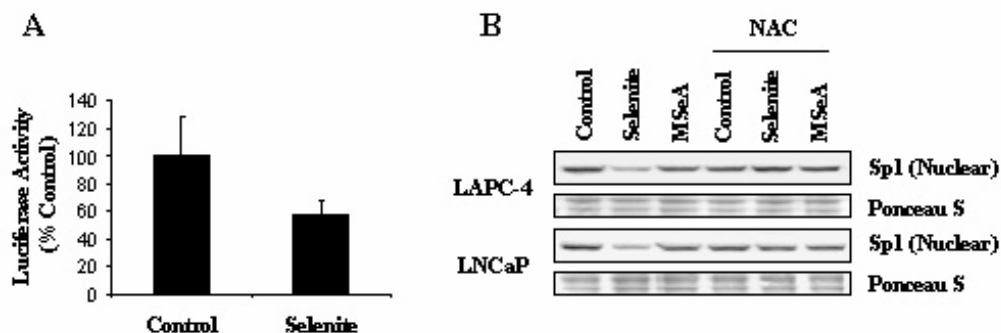


Figure 7 Effects of selenite and MSeA on Sp1. **A**, LAPC-4 cells were co-transfected with the Sp1 reporter vector, pSp1-luc, and pSV40-ren and then treated with 10 μ M selenite for 8 hours. Luciferase activity was normalized to renilla and expressed as percent of control. Values represent the mean \pm SD for 3 experiments. **B**, Western blot analysis of Sp1 protein expression in the nuclear extracts of LAPC-4 and LNCaP cells exposed to selenite or MSeA for 8 hours with or without NAC pre-treatment. Ponceau S stained bands were used to show equal loading of samples.

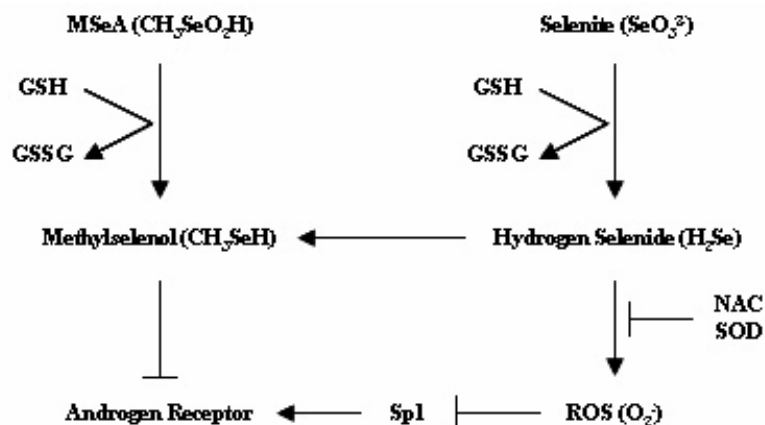


Figure 8 Schematic illustration showing the inhibition of AR expression by selenite and MSeA in prostate cancer.

Effects of selenite on the growth of LAPC-4 xenograft tumors in nude mice

In vivo pilot studies were performed in male and female nude mice with subcutaneous LAPC-4 xenograft tumors. Female mice were used because under the selective pressure of androgen deprivation, LAPC-4 tumors reproducibly evolve to a hormone refractory state, thereby providing a model for the study of androgen-independence. Once tumors reached approximately 100 mm^3 in size the mice were treated with 2 mg/kg selenite three times per week. Selenite treated mice showed significant tumor growth inhibition compared to untreated control animals (Fig. 9). In addition, there was no observed depression of body weight in selenite treated mice relative to control mice (data not shown). The results demonstrate that selenite can delay the growth of both androgen-dependent and androgen-independent LAPC-4 tumors and the systemic toxicity of selenite may not mitigate potential therapeutic efficacy. We are now repeating these experiments with larger treatment groups in an attempt to correlate the growth delay caused by selenite with changes in AR expression and serum PSA levels.

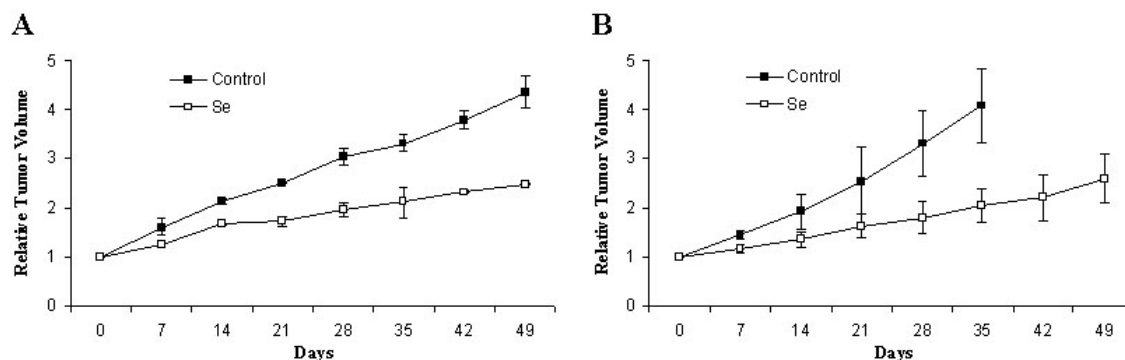


Figure 9 Effects of selenite on the growth of LAPC-4 xenograft tumors in **A**, male and **B**, female mice. Mice were treated with 2 mg/kg selenite i.p. three times per week. Tumor volume was calculated weekly (tumor volume = $\pi/6 \times \text{length} \times \text{width} \times \text{height}$).

Task 2. Show that primary prostate epithelial cells are more sensitive to selenite-induced apoptosis than normal cells and correlate these findings with differential expression of antioxidants (GSH and MnSOD) and Bcl-2 family members.

This task was completed in year 1. See publication “Tumor-selective killing by selenite in patient matched pairs of normal and malignant prostate cells.”

Task 3. Study the effect of combining selenite with radiation on apoptosis and overall tumor cell killing in primary prostate cancer cell strains and LAPC-4 cells *in vitro* and *in vivo*.

We showed that selenite was able to sensitize prostate cancer cells to γ -irradiation *in vitro* during year 1. See publication “Redox modulation of human prostate carcinoma cells by selenite increases radiation-induced cell killing.” We are now working on combining selenite with single and fractionated radiation dosage regimens *in vivo* using the LAPC-4 xenograft model.

Key Research Accomplishments (year 2 in bold)

Selenite inhibited cell growth and induced apoptosis in androgen-dependent LAPC-4 human prostate cancer cells *in vitro*

Primary cultures of normal prostate epithelial cells were more resistant to selenite-induced apoptosis than LAPC-4 cells

Selenite-induced apoptosis in LAPC-4 cells was associated with decreased GSH:GSSG and bcl-2:bax ratios

Inhibition of cell growth by selenite in LAPC-4 and LNCaP cells was associated with decreased androgen receptor expression and activity

The inhibition of AR signaling by selenite occurs by a redox-dependent mechanism that is distinct from methylseleninic acid

The inhibition of AR expression and activity by selenite occurs via a redox-mechanism involving GSH, superoxide, and Sp1

Selenite inhibited the growth of androgen-dependent and androgen-independent LAPC-4 xenograft tumors in nude mice without systemic toxicity

Using patient-matched pairs of normal and malignant prostate cells we have shown that prostate cancer-derived cells are more sensitive to selenite-induced apoptosis than the corresponding normal cells

Normal cells had increased MnSOD expression and SOD activity compared to cancer cells

Increasing MnSOD activity in cancer cells protected against selenite-induced apoptosis

Increased MnSOD expression in normal cells may be a predictive marker for the therapeutic response to selenite

LAPC-4 and DU 145 cells pretreated with selenite showed increased sensitivity to γ -irradiation

Reportable Outcomes (year 2 in bold)

Husbeck, B, Peehl, DM, Knox, SJ. Redox modulation of human prostate carcinoma cells by selenite increases radiation-induced cell killing. *Free Rad Biol Med* 38: 50-57, 2005.

Husbeck B, Nonn L, Peehl DM, Knox SJ. Tumor-selective killing by selenite in patient-matched pairs of normal and malignant prostate cells. *Prostate* 66: 218-225, 2006.

Husbeck B, Bhattacharyya RS, Feldman D, and Knox SJ. Inhibition of androgen receptor signaling by selenite and methylseleninic acid in prostate cancer cells: Two distinct mechanisms of action. Submitted to *Mol Cancer Ther* 2006.

Stanford Patent Application (S03-309) Methods for Treating A Neoplastic Disease In a Subject Using Inorganic Selenium-Containing Compounds

Conclusions

Recent studies have shown that the selenium-based compound MSeA can disrupt AR signaling in prostate cancer cells. We have found that selenite can inhibit AR expression and activity in LAPC-4 and LNCaP prostate cancer cells as well, but through a different mechanism. Upon entering the cell, selenite consumes reduced GSH and generates superoxide radicals. Pre-treatment with NAC, a GSH precursor, blocked the down-regulation of AR mRNA and protein expression by selenite and restored AR ligand binding and PSA expression to control levels. MSeA reacts with reduced GSH within the cell, however, NAC did not effect MSeA-induced down-regulation of AR and PSA. The superoxide dismutase mimetic, MnTMPyP, was also found to prevent the decrease in AR expression caused by selenite, but not by MSeA. A Sp1 binding site in the AR promoter is a key regulatory component for its expression. Selenite decreased Sp1 expression and activity, whereas MSeA did not. The inhibition of Sp1 by selenite was reversed in the presence of NAC. In conclusion, we have found that selenite and MSeA disrupt AR signaling by distinct mechanisms. The inhibition of AR expression and activity by selenite occurs via a redox-mechanism involving GSH, superoxide, and Sp1.

The development of prostate cancer and its progression to a hormone-refractory state is highly dependent on AR expression. The finding that hormone-refractory prostate cancer is not associated with a loss of AR expression, but is instead characterized by the presence of a functionally intact, although frequently overexpressed or mutated AR, has heightened interest in the AR as a therapeutic target. Current hormone therapy for prostate cancer only reduces circulating androgen levels or blocks agonist binding to the AR without decreasing AR levels. Strategic targeting of the AR with ribozymes, antisense oligomers, and small interfering RNAs has been shown to significantly inhibit prostate cancer growth both *in vitro* and *in vivo* (9-11). Thus, the ability of different selenium compounds to reduce AR levels in prostate cancer has many therapeutic indications and needs to be explored further. However, it is not yet known whether the concentrations of selenium necessary for inhibition of AR signaling can be safely achieved in human tissues. Hopefully ongoing animal studies and future human trials will address questions regarding optimal dosage regimens, and enable the full potential clinical utility of selenium compounds in prostate cancer to be realized.

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